Acid Sphingomyelinase–Deficient Human Lymphoblasts and Mice Are Defective in Radiation-Induced Apoptosis

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Summary

Stress is believed to activate sphingomyelinase to generate ceramide, which serves as a second messenger in initiating the apoptotic response. Conclusive evidence for this paradigm, however, is lacking. In the present study, we used a genetic approach to address this issue directly. We show that lymphoblasts from Niemann–Pick patients, which have an inherited deficiency of acid sphingomyelinase activity, fail to respond to ionizing radiation with ceramide generation and apoptosis. These abnormalities are reversible upon restoration of acid sphingomyelinase activity by retroviral transfer of human acid sphingomyelinase cDNA. Acid sphingomyelinase knockout mice also expressed defects in radiation-induced ceramide generation and apoptosis in vivo. Comparison with p53 knockout mice revealed that acid sphingomyelinase–mediated apoptosis and p53-mediated apoptosis are likely distinct and independent. These genetic models provide definitive evidence for the involvement of acid sphingomyelinase in one form of stress-induced apoptosis.

Introduction

The sphingomyelin pathway is a ubiquitous, evolutionarily conserved signaling system initiated by hydrolysis of sphingomyelin to generate the second messenger ceramide. Two forms of sphingomyelinase, distinguishable by the pH optima, are capable of initiating signaling. Acid sphingomyelinase (pH optimum 4.5–5.0) was originally identified as a lysosomal hydrolase required for turnover of cellular membranes (for review see Kolesnick, 1991). However, Kronke and coworkers proposed that this enzyme was also targeted to the plasma membrane and signaled in response to activation of the 55 kDa tumor necrosis factor (TNF) receptor (Wiegmann et al., 1994). Activation of acid sphingomyelinase has also now been associated with signaling via Fas, CD28, and the interleukin-1 (IL-1) receptor (Cifone et al., 1993; Boucher et al., 1995; Liu and Anderson, 1995). Human acid sphingomyelinase is the product of a single gene, although alternative processing of the primary transcript allows for the generation of multiple forms (Schuchman et al., 1991, 1992). Inherited mutations of the human acid sphingomyelinase gene lead to enzyme deficiency and the genetic disorder known as Niemann–Pick disease (NPD) (Brady et al., 1966; Schneider and Kennedy, 1967).

Neutral sphingomyelinase (pH optimum 7.4) was originally defined as a Mg^{2+}-dependent enzyme localized to the outer leaflet of the plasma membrane (Rao and Spence, 1976; Yedger and Gatt, 1976). However, a Mg^{2+}-independent isoform of neutral sphingomyelinase that localizes to the cytoplasm has recently been identified (Okazaki et al., 1989, 1994). While the neutral sphingomyelinase has not yet been characterized at the molecular level, it nonetheless seems to represent the product of a distinct gene or genes, since acid sphingomyelinase knockout mice contain physiologic levels of neutral sphingomyelinase activity (Horinouchi et al., 1995). Neutral sphingomyelinase activation has been demonstrated in response to cellular stimulation with TNFα (Wiegmann et al., 1994), anti-Fas antibody (Tepper et al., 1995; Cifone et al., 1995), and vitamin D (Okazaki et al., 1989, 1994). It has also been suggested that neutral sphingomyelinase signals in response to IL-1β (Mathias et al., 1993) and ionizing radiation (Haimovitz-Friedman et al., 1994).

A number of direct targets for ceramide action have been identified. One well-defined target is a ceramide-activated protein kinase (CAPK), which is a member of an emerging family of proline-directed serine/threonine protein kinases (Liu et al., 1994). The activity of CAPK is enhanced by treatment of intact cells or isolated membranes with TNFα, IL-1β, ceramide analogs, and bacterial sphingomyelinases (Zhang and Kolesnick, 1995). CAPK was shown to phosphorylate and activate Raf1, coupling cell surface receptors to the extracellular signal–regulated protein kinase (ERK) cascade (Yao et al., 1995). Another potential target for ceramide action is ceramide-activated protein phosphatase (CAPP), a member of the heterotrimeric protein phosphatase 2A family (Dobrowsky and Hannun, 1992). Although substrates for CAPP have yet to be identified, CAPP was reported to translocate to membrane upon cellular stimulation with TNFα (Dobrowsky and Hannun, 1993). Recent investigations suggest that ceramide may also directly activate the protein kinase C isoform ζ (Lozano et al., 1994; Muller et al., 1995) and link cytokine receptors to nuclear factor κB activation. The putative guanine-nucleotide exchange factor Vav, a potential activator of Ras and related proteins in hematopoietic cells (Gulbins et al., 1994), may also serve as a ceramide target.

Apoptosis is a preprogrammed death pathway that is constitutively expressed in many cells, albeit in an inactive form. Although the upstream signaling of apoptosis remains uncertain, ceramide has been implicated as the second messenger for a variety of stress

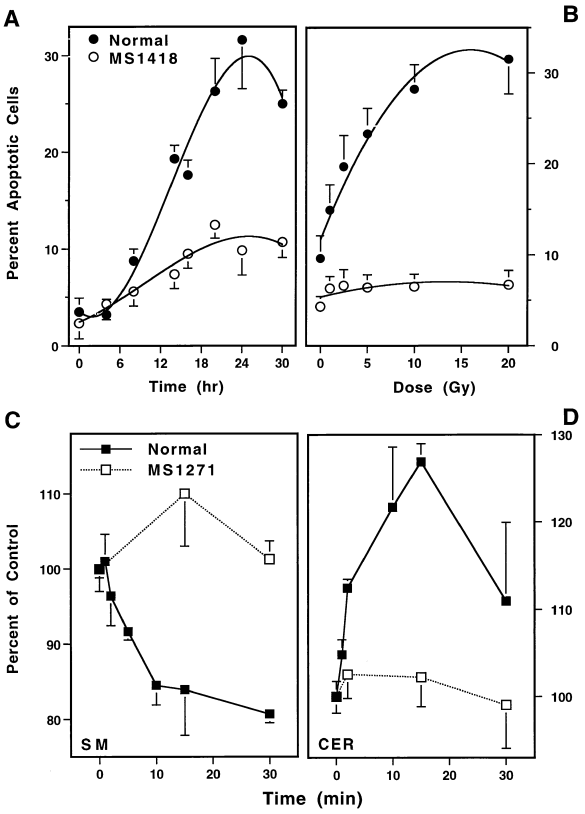


Figure 1. Radiation Induces Sphingomyelin Hydrolysis to Ceramide and Apoptosis in Normal Lymphoblasts, but Not in Lymphoblasts from NPD Patients

(A) Normal lymphoblasts or MS1418 NPD lymphoblasts were irradiated with 20 Gy and incubated at 37°C for the indicated times. Morphologic changes of nuclear apoptosis were quantified by staining with the DNA-specific fluorochrome bis-benzimide. Cells with condensation of chromatin, its compaction along the periphery of the nucleus, or segmentation of the nucleus into three or more chromatin fragments were considered apoptotic. A minimum of 500 cells were scored for the incidence of apoptosis. The data (mean ± SEM) represent two independent determinations from three separate experiments.

(B) The dose dependence of radiation-induced apoptosis was assessed in both types of lymphoblasts at 24 hr. Apoptotic cells were quantified as in (A). The data (mean ± SEM) represent duplicate determinations from four separate experiments.

(C) Normal or MS1271 NPD lymphoblasts were labeled to isotopic equilibrium with medium containing [^{3}H]choline (1 μCi/ml), irradiated with 20 Gy, and incubated at 37°C for the times indicated. Lipids were extracted with chloroform:methanol:1N HCl (100:100:1, v/v/v) and subjected to mild alkaline hydrolysis to remove glycerolipids, and sphingomyelin (SM) was resolved by thin-layer chromatography. Sphingomyelin levels were determined by lipid scintillation spectrometry. The values represent mean ± SEM of independent triplicate determinations from two separate studies.

(D) Unlabeled lymphoblasts were handled as in (C) and ceramide (CER) levels were quantified by the diacylglycerol kinase assay as described in Experimental Procedures. The values represent mean ± SEM of independent triplicate determinations from three separate studies with control lymphoblasts and five experiments with MS1271 NPD lymphoblasts.

stimuli, including TNFα, Fas ligand, ionizing radiation, heat shock, ultraviolet light, and oxidative stress (Obeid et al., 1993; Cifone et al., 1993; Jarvis et al., 1994; Fuks et al., 1995; Haimovitz-Friedman et al., 1994; Gulbins et al., 1995; Verheij et al., 1996; Jarvis et al., 1995). However, in most of these experimental systems, definitive proof that ceramide generation is the primary mediator of the apoptotic response is lacking. For the most part, evidence has been circumstantial and derived from studies that showed that stress induced rapid ceramide generation and that the increase in ceramide levels correlated closely with the apoptotic response (Verheij et al., 1996; Hannun and Obeid, 1995). Treatment with bacterial sphingomyelinase or with ceramide analogs mimicked the apoptotic response, while elevation of the level of other lipid second messengers, including arachidonic acid, phosphatidic acid, and 1,2-diacylglycerol, failed to initiate apoptosis.

In the present studies, we used a genetic approach to delineate the role of acid sphingomyelinase in the apoptotic response to ionizing radiation. We show that lymphoblasts from patients with NPD exhibit a major defect in radiation-induced ceramide generation and apoptosis. These defects are reversible upon restoration of acid sphingomyelinase activity by retroviral transfer of the human acid sphingomyelinase cDNA. Further, mice rendered deficient in acid sphingomyelinase activity by targeted gene disruption also express defects in radiation-induced ceramide generation and apoptosis. A comparison of tissue responses in acid sphingomyelinase and p53 knockout mice exposed to whole-body irradiation revealed that acid sphingomyelinase– and p53-mediated apoptosis are distinct and independent. These genetic models define an obligatory role for ceramide generation in signaling of at least one form of the apoptotic response.

Results

Radiation Induces Apoptosis in Normal Lymphoblasts, but Not in Lymphoblasts from NPD Patients

Previous studies proposed that ionizing radiation induces apoptosis in several cell lines via sphingomyelinase activation and the generation of ceramide (Haimovitz-Friedman et al., 1994; Verheij et al., 1996). Figure 1A shows that exposure of Epstein–Barr virus (EBV)-transformed normal human lymphoblasts to a radiation dose of 20 Gy resulted in time-dependent apoptosis as defined by morphologic changes of chromatin condensation and compaction and nuclear segmentation. Apoptosis was detected by 8 hr and was maximal by 24 hr. As little as 1 Gy was effective, and a peak effect was achieved with 20 Gy (Figure 1B). In contrast, the EBV-transformed NPD lymphoblast lines MS1418 (Figures 1A and 1B) and MS1271 (data not shown) did not demonstrate a significant apoptotic response. The differences in the apoptotic response could not be attributed to altered cell proliferation, because the growth rates of NPD and normal lymphoblasts did not differ appreciably (data not shown).

Radiation Induces Ceramide Generation in Normal Lymphoblasts, but Not in Lymphoblasts from NPD Patients

To determine whether these differences correlated with differences in ceramide generation, we exposed normal

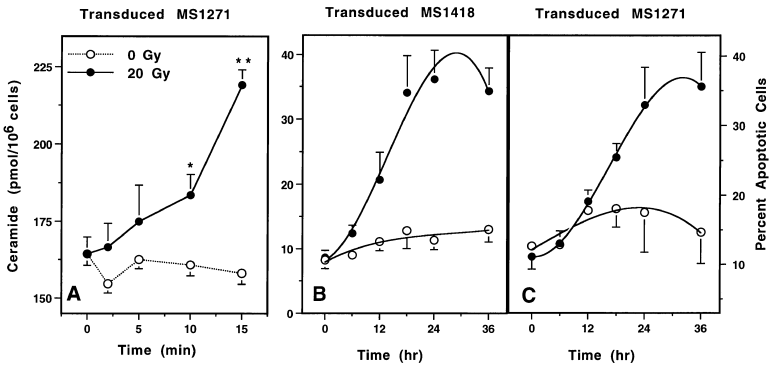


Figure 2. Retroviral Transduction of the Acid Sphingomyelinase cDNA Restores Radiation-Induced Ceramide Generation and Apoptosis to NPD Lymphoblasts

(A) Retroviral transduction was performed by coincubation for 48 hr with amphotropic packaging cell lines that secrete acid sphingomyelinase–containing retrovirus as described in the Experimental Procedures. MS1271 NPD lymphoblasts transduced with the acid sphingomyelinase cDNA were incubated for 24 hr in serum-free medium and then irradiated at 20 Gy. Ceramide levels were determined as in Figure 1D at the times indicated. The data (mean ± SEM) represent two independent determinations from two separate experiments. Single asterisk, $p < 0.05$; double asterisk, $p < 0.005$.

(B and C) NPD lymphoblasts were infected with acid sphingomyelinase–containing retrovirus as in (A) and irradiated with 20 Gy. Apoptosis was quantified by staining with bis-benzimide as in Figure 1. Values represent the mean ± SEM of independent determinations from three (MS1418) or four (MS1271) separate experiments.

and NPD lymphoblasts to ionizing radiation and measured sphingomyelin hydrolysis to ceramide. Lymphoblasts from two normal individuals displayed acid sphingomyelinase activity of 6.0 ± 0.2 nmol sphingomyelin hydrolyzed per milligram of protein per hour (mean ± range). The NPD lines MS1418 and MS1271 expressed only 2%–3% residual acid sphingomyelinase activity of 0.19 and 0.13 nmol sphingomyelin hydrolyzed per milligram of protein per hour, respectively, consistent with previously published data (Suchi et al., 1992). Figure 1C shows that a dose of 20 Gy induced a rapid reduction in sphingomyelin content in normal lymphoblasts from a baseline level of 440 pmol per 10^6 cells. This effect was detected by 2 min and persisted for 30 min ($p < 0.001$ versus basal level). The reduction in sphingomyelin content was accompanied by a near quantitative increase in ceramide above a basal level of 180 pmol per 10^6 cells (Figure 1D). Ceramide elevation was detected by 1–2 min and was maximal at 15 min ($p < 0.001$ versus basal level). Consistent with the deficiency in acid sphingomyelinase activity, MS1271 lymphoblasts had elevated basal levels of sphingomyelin of 565 pmol per 10^6 cells. These cells did not respond to 20 Gy radiation at any time between 0 and 30 min with a decrease in sphingomyelin (Figure 1C) or an increase in ceramide content (Figure 1D). Similar results were obtained with the NPD line MS1418 (data not shown), which exhibits a higher baseline sphingomyelin content of 1110 pmol per 10^6 cells. It should be noted that the lack of response was specific for deficiency of acid sphingomyelinase, since these NPD lymphoblasts contain a normal level of neutral sphingomyelinase activity of 2.2 nmol sphingomyelin hydrolyzed per milligram of protein per hour.

Retroviral Transfer of Acid Sphingomyelinase cDNA Restores Radiation-Induced Ceramide Generation and Apoptosis to NPD Lymphoblasts

To demonstrate a role for acid sphingomyelinase in induction of apoptosis by ionizing radiation, we restored acid sphingomyelinase activity to the NPD lymphoblasts by retroviral transfer. Retroviral transduction of the human acid sphingomyelinase cDNA into the NPD lymphoblast lines increased acid sphingomyelinase activity 17-fold in MS1418 cells to 3.24 nmol sphingomyelin hydrolyzed per milligram of protein per hour and 8-fold in MS1271 cells to 1.03 nmol sphingomyelin hydrolyzed per milligram of protein per hour. Routinely, expression was greater in line MS1418 than in MS1271. Introduction of the acid sphingomyelinase cDNA restored radiation-induced ceramide generation to MS1271 (Figure 2A) and MS1418 cells (data not shown) and apoptosis (Figures 2B and 2C). In contrast, retroviral transduction of an irrelevant cDNA for the enzyme arylsulfatase did not restore radiation-induced ceramide generation or apoptosis to MS1271 or MS1418 cells (data not shown).

Acid Sphingomyelinase Knockout Mice Have a Defect in Radiation-Induced Apoptosis

In previous studies, we reported that ionizing radiation induced marked apoptotic changes in the lungs of C3H/HeJ mice in vivo, as measured by the DNA terminal transferase nick-end translation method (TUNEL) assay (Fuks et al., 1995). The walls of pulmonary alveoli consist of an extensive network of capillaries, which allow for efficient gas exchange, and type I and II pneumocytes for support. The prior investigations showed that the apoptotic response in the irradiated lung was largely confined to microvascular endothelial cells. This effect was detected by 6 hr after irradiation and was maximal by 10 hr. Thereafter, apoptotic cells were phagocytized by alveolar macrophages and could no longer be detected by 24 hr.

To determine whether ceramide might play a role in radiation-induced apoptosis in this mouse lung model, we exposed wild-type C3H/HeJ mice to 10 Gy whole-body radiation and measured ceramide content in extracts of lung tissue. Ceramide generation above a basal level of 280 nmol per gram of lung tissue was detected as early as 5 min after irradiation and was maximal by 30 min (data not shown). Thus, ceramide generation preceded the onset of apoptosis. The effect of radiation on ceramide generation was dose dependent. As little as 7.5 Gy was effective, and a maximal effect to 232% of control was demonstrated after 20 Gy at 30 min (data not shown). This dose range is consistent with that previously shown to induce apoptosis in lungs of C3H/HeJ mice (Fuks et al., 1995).

To analyze the role of acid sphingomyelinase in radiation-induced ceramide generation and apoptosis in

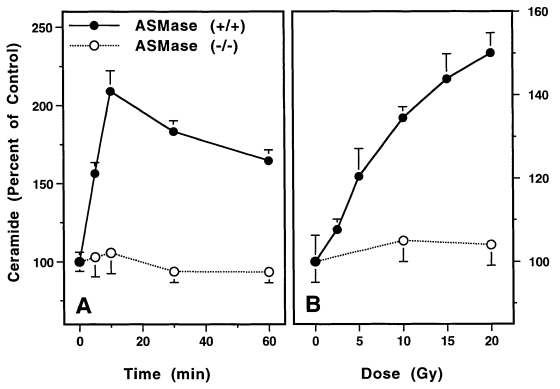


Figure 3. Radiation Increases Ceramide Levels in Lungs of 129/Sv (Acid Sphingomyelinase +/+) but Not in the Knockout (Acid Sphingomyelinase −/−) Mice

(A and B) Male 129/Sv (acid sphingomyelinase [ASMase] +/+) and knockout (ASMase −/−) mice received whole-body radiation at 10 Gy (A) or at varying doses for 30 min (B) and at the indicated times were sacrificed by cervical dislocation. The lungs were dissected and homogenized in 8 vol (w/v) of ice-cold PBS, and lipids were extracted with chloroform:methanol (2:1, v/v). Ceramide levels were measured by fluorescence spectrometry after derivitization with o-phthaldehyde, as described in Experimental Procedures. The values represent mean ± SEM of duplicate determinations from two separate experiments.

vivo, we employed an acid sphingomyelinase–deficient mouse model. Acid sphingomyelinase knockout mice were generated by targeted disruption of the acid sphingomyelinase gene of 129/Sv mice within exon 2 (Horinouchi et al., 1995). Homozygous acid sphingomyelinase knockout mice appear normal at birth and develop routinely until about 4 months of age, when they begin to show signs of neurologic disease including ataxia, tremors, and loss of appetite (Otterbach and Stoffel, 1995; Horinouchi et al., 1995). Affected animals have no detectable acid sphingomyelinase activity and accumulate sphingomyelin and cholesterol in various tissues as their disease progresses (Horinouchi et al., 1995). For the studies reported in this manuscript, we analyzed the acid sphingomyelinase knockout mice within 3 weeks of weaning, a time at which no disease manifestations are detected.

Similar to C3H/HeJ mice, radiation induced time- and dose-dependent ceramide generation above a basal level of 225 nmol per gram of tissue in the lungs of the wild-type 129/Sv mice (Figure 3). However, radiation did not increase ceramide content in the lungs of the acid sphingomyelinase knockout mice. Exposure to 20 Gy induced extensive apoptosis in the lungs of wild-type 129/Sv mice after 10 hr (Figure 4, upper left panel). Higher magnification of these tissue specimens showed that apoptosis occurred primarily in the endothelium (Figure 4, lower left panel). In contrast, acid sphingomyelinase–deficient mice did not exhibit significant pulmonary apoptosis in response to 20 Gy (Figure 4, right panels). Increasing the radiation dose up to 30 Gy failed to generate an apoptotic response in the lungs of the acid sphingomyelinase–deficient mice.

The induction of apoptosis as a consequence of radiation was also evaluated in thymic and splenic tissue from 129/Sv and C3H/HeJ mice. Both strains yielded identical results. Apoptosis occurred more rapidly in thymic than lung tissue, and the response was biphasic. A rapid phase, detected by 1 hr with 7.5 Gy, peaked at 3 hr and was followed by a slower response (Figure 5A). As little as 2 Gy was effective, and a maximal effect was achieved with 10 Gy. As compared to the lung, the acid sphingomyelinase knockout mice demonstrated a less comprehensive defect in radiation-induced apoptosis in thymic tissue. Unirradiated thymic tissue from normal and knockout animals manifested a baseline 4%–5% incidence of apoptosis (data not shown). Figure 6 shows that a dose of 5 Gy induced substantial apoptosis in the thymic cortex of 129/Sv mice at 2.5 hr after irradiation. Many of the apoptotic cells formed clusters of 5–8 cells surrounded by normal-appearing cells. In contrast, minimal apoptosis was detected in the thymic cortex of the acid sphingomyelinase knockout mice. When 2000 129/Sv thymic cells were counted in four high power fields (Figure 5B), a total of 652 (33%) showed apoptotic changes. In contrast, only 346 (17%) of 2000 cells in the thymic cortex of the acid sphingomyelinase knockout mouse were apoptotic ($p < 0.0001$). Statistically significant differences in thymic apoptosis were also observed between 129/Sv and knockout mice at 4 Gy and 7.5 Gy (Figure 6C). After 4 hr, there was a rapid increase in the incidence of apoptosis in both strains. A diffuse pattern gradually developed throughout the thymic cortex, and detectable differences diminished, perhaps owing to overcrowding by the late-responding apoptotic cells. Differences were no longer detected by 10 hr. Qualitatively similar results were observed in the spleen (Figure 5B).

Comparison of p53 and Acid Sphingomyelinase Knockout Mice

Because of the variation in tissue responses of the acid sphingomyelinase knockout mice, we studied p53 knockout mice, also known to be defective in radiation-induced apoptosis. Figure 7 shows apoptotic responses to 20 Gy in wild-type C57Bl/6 mice and the p53 knockout mice derived from this strain. Both the cortex (Figure 7, upper left panel) and medulla (data not shown) of the wild-type thymus demonstrated diffuse apoptosis at 10 hr. In contrast, only 13% of the thymic cells of the p53 knockout mice expressed apoptotic changes (Figure 7, upper right panel), appearing mostly in clusters of 5–8 cells. Increasing the radiation dose to 30 Gy failed to increase the apoptotic response in the thymus of the p53-deficient mice. These in vivo studies agree with previous ex vivo studies, which reported a 10%–20% incidence of radiation-induced apoptosis in thymocytes derived from p53 knockout mice (Strasser et al., 1994; Lowe et al., 1993).

In contrast to the thymus, lungs of p53-deficient mice (Figure 7, lower right panel) exhibited a normal apoptotic response at 10 hr after exposure to 20 Gy. The effect appeared identical to that observed in wild-type C57Bl/6 mice (lower left panel) and in 129/Sv (Figure 4) and C3H/HeJ mice (Fuks et al., 1995). It should again be noted that the lungs of acid sphingomyelinase–deficient mice did not undergo apoptosis in response to radiation (Figure 4).

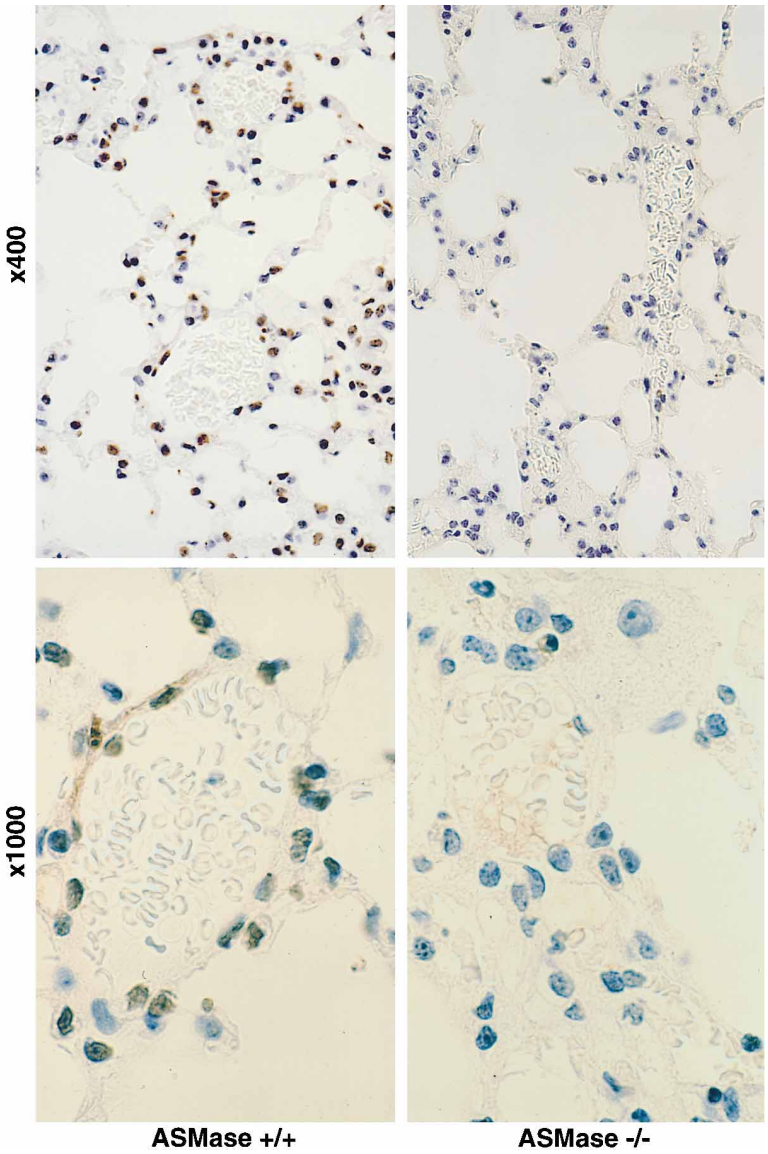


Figure 4. Radiation Induces Apoptosis in Lungs of 129/Sv (Acid Sphingomyelinase +/+) but Not in the Knockout (Acid Sphingomyelinase −/−) Mice

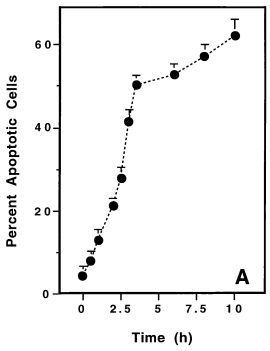
Lung specimens from 129/Sv (acid sphingomyelinase [ASMase] +/+) and knockout (ASMase −/−) mice were obtained 10 hr after exposure to 20 Gy whole-body irradiation. Tissues were fixed in formalin and paraffin embedded, and 5 μm sections were used for TUNEL assays. Apoptotic nuclei are identified by brown-yellow staining, a product of the diaminobenzidine chromogen used. In contrast, normal nuclei stain blue owing to counterstaining with hematoxylin. Note the intense TUNEL signal in the nuclei of endothelial cells of small blood vessels and capillaries and, occasionally, in alveolar pneumocytes in the lung of the 129/Sv mouse (left upper and lower panels). The majority of capillaries and small blood vessels and all pneumocytes of the acid sphingomyelinase knockout mice display negative TUNEL signals (right upper and lower panels). Original magnification: upper panels, 400×; lower panels, 1000×. This experiment represents one of four similar studies.

To explore the sensitivity of other p53-deficient tissues to radiation-induced apoptosis, we exposed knockout mice to whole-body radiation (20 Gy). Apoptotic responses were observed at 10 hr in the pleura, endocardium, pericardium, and the germinal centers of the spleen (data not shown). Apoptotic responses were not observed in liver, kidney, brain, skin, the myocardium, and striated muscle. This pattern of tissue response was identical to that observed in C57Bl/6, 129/Sv, and C3H/HeJ mice when exposed to a similar radiation dose (data not shown). However, the radiation response in acid sphingomyelinase knockout mice differed. These mice did not develop apoptosis in endocardium, pericardium, or pleura (data not shown).

Discussion

The present studies address the role of ceramide generation via acid sphingomyelinase in induction of apoptosis using two separate genetic models. Lymphoblast cell lines from NPD patients demonstrated a defect in radiation-induced apoptosis, which was reversible upon restoration of acid sphingomyelinase activity. Defects in radiation-induced apoptosis were also observed in tissues of acid sphingomyelinase knockout mice, which contain physiologic levels of neutral sphingomyelinase activity (Horinouchi et al., 1995). It should be noted that humans born with this mutation, as well as the acid sphingomyelinase knockout mice, present no developmental defects at the time of birth. Hence, the signaling systems for this form of stress-induced apoptosis and developmental apoptosis seem to be distinct. Consistent with this notion, IL-1β-converting enzyme, p53, and poly(ADP-ribose) polymerase knockout mice are also born without apparent developmental defects (Kuida et al., 1995; Kemp et al., 1993; Wang et al., 1995).

The present investigations indicate that acid sphingomyelinase–dependent apoptosis may be distinct from p53-dependent apoptosis. In some tissues, such as the endothelium of the lung and heart and the mesothelium



B

Mice	Tissues	4Gy	5 Gy	7.5 Gy
129/SV	Thymus	558 (27.9%)	652 (32.6%)	737 (36.9%)
ASMase -/-		240 (12.0%)*	346 (17.3%)*	565 (28.2%)*
129/SV	Spleen	286 (14.3%)	470 (23.5%)	560 (28.0%)
ASMase -/-		185 (9.20%)*	323 (16.1%)*	397 (19.8%)*

* p<0.001 vs. 129/SV

Figure 5. Radiation Induces Apoptosis in Thymic Tissue from Wild-Type and Acid Sphingomyelinase Knockout Mice

(A) Radiation induces a time-dependent increase in apoptosis in the thymus of C3H/HeJ mice. C3H/HeJ mice received whole-body radiation at 7.5 Gy and were sacrificed at the times indicated. Apoptosis in thymic tissue was measured by TUNEL assay as described in Figure 4. A minimum of 1000 cells were scored for the incidence of apoptosis. The data (mean ± range) represent duplicate determinations from one representative of three separate experiments.

(B) Quantitation of apoptosis in thymic and splenic tissue from 129/Sv (acid sphingomyelinase [ASMase] +/+) and knockout (ASMase −/−) mice at 2.5 hr postirradiation. We counted 2000 cells in four high power fields (400×). Numbers indicate apoptotic cells, and the percentage of apoptotic cells is shown in parentheses. Differences between 129/Sv and ASMase −/− were evaluated by χ^2, and at each dose tested, $p < 0.001$.

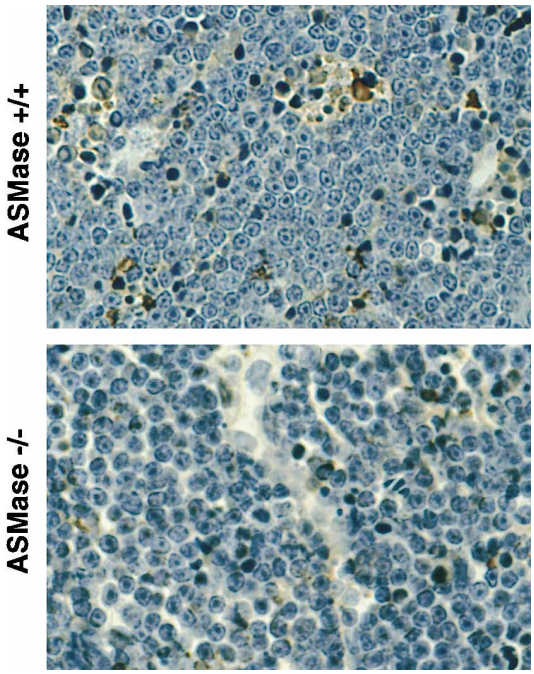


Figure 6. Reduction in Radiation-Induced Apoptosis in Thymic Tissue from Acid Sphingomyelinase Knockout Mice

Thymic samples from 129/Sv (acid sphingomyelinase [ASMase] +/+) and knockout (ASMase −/−) mice were obtained 2.5 hr after exposure to 5 Gy whole-body irradiation and handled as in Figure 4. We used 5 μm sections for TUNEL assays, and apoptotic nuclei were identified as specified in Figure 4. An intense TUNEL signal was noted in the nuclei of a proportion of thymocytes in the cortical region of 129/Sv treated mice, occasionally forming small clusters of apoptotic cells (top). In certain instances, TUNEL staining was identified as a dark brown-to-blue reaction due to nuclear condensation of cells undergoing apoptosis. The percentage of positive TUNEL thymocytes was reduced in the acid sphingomyelinase knockout mice, in which cluster formation was rare and only scattered apoptotic cells were observed (bottom). Original magnification, 400×. This experiment represents one of three similar studies.

of the pleura and pericardium, radiation-induced apoptosis appears primarily dependent on acid sphingomyelinase and for the most part independent of p53. In contrast, thymic apoptosis appears highly dependent on p53 and dependent to a lesser degree on acid sphingomyelinase. Hence, radiation appears capable of activating two apparently independent signaling mechanisms for induction of apoptosis. These signaling mechanisms may be topologically separate, as the sphingomyelin pathway appears to be membrane based, whereas p53-dependent apoptosis is considered secondary to DNA damage.

Prior investigations support the concept of apoptotic signaling mechanisms that do not involve p53. In this regard, thymic cells from p53 knockout mice demonstrated no resistance to glucocorticoid-, phorbol ester-, or ionomycin-induced apoptosis (Strasser et al., 1994). Further, a portion of thymic cells from the p53 knockout mice were found to be radiosensitive (Strasser et al., 1994; Lowe et al., 1993; Lowe, 1995). T cells from lymphomas arising spontaneously in the thymus of $p53^{-/-}$ mice also do not display radiation resistance. Seki et al. (1994) examined the p53 dependence of this radiation response in detail and reported that specific subpopulations of human peripheral blood lymphocytes underwent p53-dependent and others p53-independent apoptosis. It remains unclear whether the small fraction of p53-deficient thymocytes that respond to radiation with apoptosis are related to the fraction of early-responding thymocytes shown in the present study to require sphingomyelinase activity to develop apoptosis.

Although the present studies demonstrate a role for acid sphingomyelinase in signaling of the apoptotic response to ionizing radiation, they do not imply that ceramide generation uniformly results in apoptosis. In fact, in some systems ceramide generation initiates differentiation (Okazaki et al., 1989) or proliferative responses (Boucher et al., 1995; Olivera et al., 1992). Kronke and coworkers offered a mechanism that clarifies how ceramide may function in diverse pathways. Using mutants of the cytoplasmic domain of the 55 kDa TNF receptor, they showed that specific receptor domains link to different sphingomyelinases. A membrane-proximal region linked the neutral sphingomyelinase to the ERK cascade

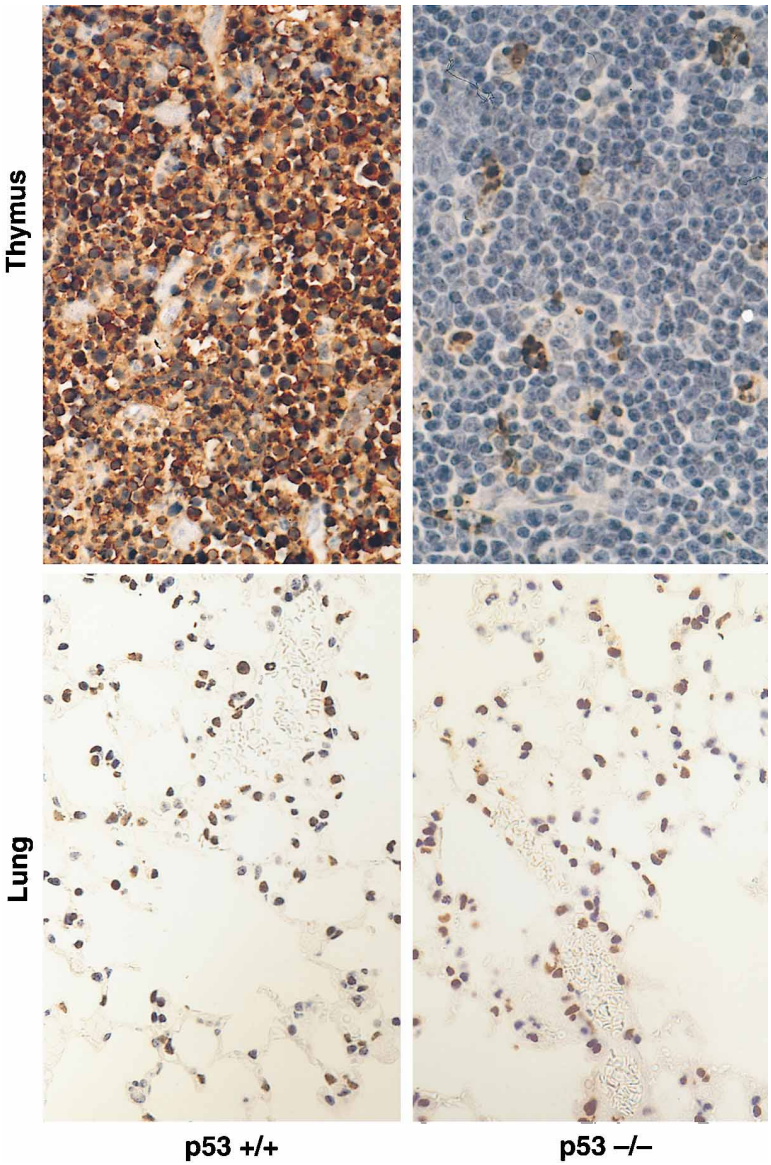


Figure 7. Radiation-Induced Apoptosis in Thymic and Lung Tissue of C57Bl/6 ($p53^{+/+}$) and p53 Knockout ($p53^{-/-}$) Mice

The upper panels show thymic specimens from C57Bl/6 ($p53^{+/+}$) and p53 knockout mice that were obtained 10 hr after exposure to 20 Gy whole-body irradiation and handled as in Figure 6 (original magnification, 400×). The lower panels show TUNEL stains of lung specimens obtained from the same mice (original magnification, 1000×).

and proinflammatory responses, whereas the carboxyl terminus, which contains the death domain, connected to acid sphingomyelinase (Wiegmann et al., 1994). These studies suggest that signaling through sphingomyelinases is compartmentalized and may lead to initiation of distinct biological processes. We speculate that radiation selects a compartment that links acid sphingomyelinase to apoptosis.

The exact mechanism by which radiation activates sphingomyelinase is unknown. The capacity of radiation to activate sphingomyelinase was demonstrated in isolated cell membrane, suggesting that DNA damage is not required (Haimovitz-Friedman et al., 1994). Once generated, ceramide rapidly signals activation of the SAPK/JNK cascade (Verheij et al., 1996) in bovine endothelial and U937 leukemia cells, two cell lines that undergo apoptosis in response to radiation. Disruption of this pathway by overexpression of dominant-negative mutants of SEK1 and c-Jun abrogated both ceramide- and radiation-induced apoptosis. In contrast, inactivation of the ERK cascade with dominant-negative mutants of Raf1 and MEK1 had no effect on induction of apoptosis. Although it is presumed that the SAPK/JNK signaling system links the cell surface to proteases that mediate the apoptotic response, information confirming this hypothesis is presently lacking.

While the present studies definitively demonstrate a requirement for acid sphingomyelinase in radiation-induced ceramide generation, we previously suggested that neutral sphingomyelinase might be involved in ceramide generation in bovine endothelial cells (Haimovitz-Friedman et al., 1994). It should be noted that in these prior studies acid sphingomyelinase activity was not measured. Further, the cell-free system used in the prior studies might not have adequately distinguished between neutral and acid sphingomyelinase activities. Now that specific reagents are becoming available, such as the acid sphingomyelinase gene and knockout

mouse, it may become possible, as in the present studies, to establish more clearly the signaling roles of different sphingomyelinases.

The demonstration that acid sphingomyelinase mediates one form of radiation-induced cell kill addresses basic concepts concerning the biological effects of ionizing radiation in mammalian cells, with a potential for clinical use. The prevailing paradigm of the lethal effects of radiation identifies double-stranded DNA breaks, produced by direct interaction with X-rays or with radiation-induced free radicals, as the critical lesions that lead to cell death (Ward, 1994; Radford, 1986; Coleman, 1993). Whereas the great majority of DNA breaks are rapidly repaired by constitutively expressed enzymatic mechanisms (Steele et al., 1989; McMillan, 1992; Hall, 1994), residual unrepaired or misrepaired breaks lead to genetic instability, increased frequency of mutations, and chromosomal aberrations (Dewey et al., 1971; Bradford, 1991). Lethal mutations or dysfunctional chromosomal aberrations eventually lead to progeny cell death, usually after several mitotic cycles (Thompson and Suit, 1969; Hurwitz and Tolmach, 1969). This model of cell kill, known as reproductive or clonogenic cell death, is enhanced in mutants deficient in DNA repair (Thompson, 1991; Harnden, 1994) and accounts for a major part of the therapeutic effects of ionizing radiation (Dewey et al., 1995). Approaches to enhancing cell kill via modulation of the initial number, structural complexity, or enzymatic repair of radiation lesions to the DNA have yielded promising results in experimental models in vitro and in vivo, but have generally failed to translate into clinical models of human cancer.

The main alternative death pathway is apoptosis. Radiation-induced apoptosis is prevalent in thymocytes, lymphocytes, and hematopoietic and embryonal cells, but is expressed at low levels in other mammalian cell types (Allan, 1992). An approach to enhance the apoptotic response may provide new opportunities to improve the clinical effects of radiation. The designation of acid sphingomyelinase as an upstream regulator of a specific death pathway suggests that it is a reasonable target for pharmacologic intervention in the apoptotic process. Intervention at this level might avoid potential complications involving modulation of final common pathways, which are necessary for multiple forms of apoptotic signaling. A better understanding of the isoforms of acid sphingomyelinase involved, and the topology of the signaling pathway, would be of help in defining an approach to pharmacologically alter enzyme activity to manipulate induction of apoptosis.

Experimental Procedures

Cell Culture

EBV-transformed lymphoblasts were established by standard techniques (Anderson and Gusella, 1984) from two NPD patients, designated MS1271 and MS1418. MS1271 is an Ashkenazi Jewish type B patient who is currently 11 years of age and neurologically intact. He carries one allele of the type B mutation, ΔR608 (Levran et al., 1991a) and another of the mutation R496L (Levran et al., 1991b). Cultured skin fibroblasts from this patient have less than 3% of normal acid sphingomyelinase activity. MS1418 was derived from a 3-year-old American boy of German ancestry who presented with massive hepatosplenomegaly and severe retardation. He was treated for recurrent pneumonia and subsequently died when he was 5 years of age. Cultured cells from patient MS1418 have <1% of normal acid sphingomyelinase activity.

EBV-transformed lymphoblasts derived from NPD patients or unaffected controls were maintained in a mixture of RPMI and DMEM media (4:1, v/v) containing 18% fetal calf serum (FBS) (GIBCO BRL). Cells were grown at 37°C in a 5% CO_2 atmosphere. Cell number and viability were assessed by trypan blue exclusion analysis. Amphotropic packaging cell lines that secrete acid sphingomyelinase–containing retrovirus (Yeyati et al., 1995) were maintained in DMEM media with 10% FBS.

For gene transfer, retrovirus packaging cells were plated in 100 mm Transwell dishes (Costar), above the 0.45 μm membrane insert, at 10×10^6 to 40×10^6 cells per dish and grown overnight. An equal number of lymphoblasts was layered beneath the Transwell insert on the following day. After 48 hr of coculture, infected lymphoblasts were removed from the dish and grown in fresh media. Expression of acid sphingomyelinase activity was maximal at 24–48 hr postinfection. Experiments were routinely performed at 24 hr postinfection.

Mice and Irradiation

We purchased 4- to 6-week-old male C3H/HeJ and C57Bl/6 ($p53^{+/+}$) mice from the Jackson Laboratories and 129/Sv and p53 knockout mice from Taconic Labs. Acid sphingomyelinase knockout mice were constructed as described previously (Horinouchi et al., 1995). In brief, embryonic stem cells derived from 129/Sv mice were transfected with an acid sphingomyelinase replacement vector containing a neomycin expression cassette inserted into exon 2 of the acid sphingomyelinase gene. Embryonic stem cell colonies containing the properly targeted acid sphingomyelinase sequences were obtained and then microinjected into blastocysts of C57Bl/6 mice. The resulting chimeric mice were used to generate the acid sphingomyelinase knockout mouse colony. The homozygous acid sphingomyelinase knockout phenotype is inherited as an autosomal recessive trait. For experiments, animals received whole-body irradiation, delivered using a Cs-137 Irradiator (Shepherd Mark-I, model 68, SN643) at a dose rate of 270 cGy/min.

The animal housing facility is approved by the American Association for Accreditation of Laboratory Animal Care and is maintained in accordance with the regulations and standards of the United States Department of Agriculture and the Department of Health and Human Services, National Institutes of Health.

Lipid Studies

On the day of an experiment, cells were resuspended into media without serum (2×10^6 cells per 0.3 ml) and irradiated. Doses are indicated in each figure. Ceramide was quantified by the diacylglycerol kinase assay as described previously (Dressler and Kolesnick, 1990). In brief, after irradiation, cells were incubated at 37°C for various lengths of time and extracted with 1 ml of chloroform:methanol:1N HCl (100:100:1, v/v/v). Lipids in the organic phase were dried under N_2 and subjected to mild alkaline hydrolysis (0.1N methanolic KOH for 1 hr at 37°C) to remove glycerophospholipids. Samples were reextracted, and lipids in the organic-phase extract were quantified via the diacylglycerol kinase reaction.

For measurement of sphingomyelin levels, cells were labeled to isotopic equilibrium with [^{3}H]choline (1 μCi/ml, specific activity 79.2 Ci/mmol; Dupont New England Nuclear) for at least three cell doublings (Dressler et al., 1992). Lipids were extracted as above, and sphingomyelin was resolved by thin-layer chromatography using chloroform:methanol:acetic acid:water (50:30:8:4) as solvent, identified by iodine vapor staining, and quantified by liquid scintillation counting. Baseline sphingomyelin mass was verified by lipid phosphorous assay (Chen et al., 1956).

Tissue ceramide content was determined by a modification of the method used for amino acid analyses (Merrill et al., 1988). After irradiation, animals were sacrificed by cervical dislocation, and tissues were weighed and homogenized in 8 vol (w/v) of ice-cold PBS. Homogenate (0.4 ml) was transferred to 16 mm × 100 mm glass tubes, and lipids were extracted with 2 ml of chloroform:methanol (2:1, v/v). Ceramide in the organic phase was measured after deacylation to sphingoid base and derivitization with o-phthaldehyde

(OPA) as described by Merrill et al. (1988). In brief, aliquots of the organic phase (250 μl) were dried under N_2, resuspended in 0.5 ml of 1N KOH in methanol, and incubated for 1 hr at 100°C to deacylate ceramide to free sphingoid bases (Van Veldhoven et al., 1989). Lipids were then dissolved in 50 μl of methanol and mixed with 50 μl of OPA reagent, which was prepared fresh daily by mixing 99 ml of 3% (w/v) boric acid in water (pH adjusted to 10.5 with KOH) and 1 ml of ethanol containing 50 mg of OPA (Sigma) and 50 μl of 2-mercaptoethanol. After incubation for 5 min at room temperature, 500 μl of methanol:5 mM potassium phosphate (pH 7.0) (90:10, v/v) was added, and the samples were clarified by brief centrifugation. Aliquots (20 μl) were quantified by reverse-phase high performance liquid chromatography using a Nova Pak C18 column (60 Å, 4 μm, 3.9 mm × 150 mm; Waters). Fluorescent lipids were eluted isocratically with methanol:5 mM potassium phosphate (pH 7.0) (90:10, v/v) at a flow rate of 0.6 ml/min and detected by spectrofluorometer (excitation wavelength 340 nm, emission wavelength 455 nm). Ceramide levels were determined by comparison to a concomitantly run standard curve of known amounts of ceramide (type III, from bovine brain sphingomyelin; Sigma). The levels of ceramide obtained by this procedure were similar to those obtained by the diacylglycerol kinase assay.

Acid Sphingomyelinase Assay

Cells (1×10^7 to 2×10^7) were pelleted (500 × g for 5 min at 4°C), washed twice with ice-cold PBS, and resuspended (1×10^6 cells per 0.3 ml) into homogenization buffer (0.2% Triton X-100). Cells were disrupted with a Tenbroeck tissue homogenizer (Bellco glass), and nuclei and debris were pelleted by centrifugation at 800 × g for 5 min.

Acid sphingomyelinase activity was measured as described previously (Maruyama and Arima, 1989). Incubations contained 30 μg of postnuclear supernatant and 15 μl of sphingomyelin substrate (9 nmol sphingomyelin mixed with 0.9 μl of [^{14}C]sphingomyelin, specific activity 56 mCi/mmol; Amersham) in acid sphingomyelinase assay buffer (250 mM Na acetate [pH 5.2], 1 mM EDTA, 0.1% Triton X-100). After 2 hr at 37°C, [^{14}C]phosphocholine was extracted with 200 μl of chloroform:methanol (1:1, v/v) and 90 μl of H_2O. Aliquots of the aqueous-phase extract were quantified by liquid scintillation counting. Acid sphingomyelinase activity is expressed as nanomoles sphingomyelin hydrolyzed per milligram of protein per hour.

Apoptosis

Morphological changes of nuclear apoptosis were visualized by staining with the DNA-binding fluorochrome bis-benzimide (Hoechst-33258; Sigma) as described previously (Bose et al., 1995). In brief, 0.5×10^6 to 2.0×10^6 cells were pelleted, washed once with PBS, and fixed in 500 μl of 3% paraformaldehyde in PBS. Thereafter, cells were resuspended into 30 μl of paraformaldehyde–PBS containing 16 μg/ml bis-benzimide. Aliquots were placed on glass slides and evaluated by fluorescence microscopy (Olympus BH-2 fluorescence microscope with a BH2-DMU2UV Dich Mirror Cube filter). A minimum of 500 cells were scored for the incidence of apoptotic chromatin changes (condensation of chromatin, its compaction along the periphery of the nucleus, and segmentation of the nucleus into more than three fragments).

Apoptosis in vivo was assessed by the DNA terminal transferase nick-end translation method, or TUNEL assay, as described previously (Fuks et al., 1995). In brief, tissue specimens were fixed overnight in 4% buffered formaldehyde and embedded in paraffin blocks. Tissue sections (5 μm thick), adherent to polylysine-treated slides, were deparaffinized by heating at 90°C for 10 min and then at 60°C for 5 min. Tissue-mounted slides were first washed with 90% and then 80% ethanol (3 min each) and rehydrated. The slides were incubated in 10 mM Tris–HCl (pH 8) for 5 min, digested with 0.1% pepsin, rinsed in distilled water, and treated with 3% H_2O_2 in PBS for 5 min at 22°C to inactivate endogenous peroxidase. After three washes in PBS, the slides were incubated for 15 min at 22°C in buffer (140 mM Na-cacodylate [pH 7.2], 30 mM Trizma base, 1 mM $CoCl_2$) and then for 30 min at 37°C in reaction mixture (0.2 U/μl terminal deoxynucleotidyl transferase, 2 nM biotin-11-dUTP, 100 mM Na-cacodylate [pH 7.0], 0.1 mM DTT, 0.05 mg/ml BSA and 2.5 mM $CoCl_2$). The reaction was stopped by transferring the slices to a bath of 300 mM NaCl, 30 mM Na citrate for 15 min at 22°C. The slides were washed in PBS, blocked with 2% human serum albumin in PBS for 10 min, rewashed, and incubated with avidin–biotin peroxidase. After 30 min at 22°C, cells were stained with the chromogen 3,3′-diaminobenzidine tetrachloride and counterstained with hematoxylin. Nuclei of apoptotic cells appear brown and granular, while normal nuclei stain blue.

Statistical Analysis

Statistical analyses were performed by Student's t test and Chi square test.

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